

University of Tennessee at Chattanooga UTC Scholar

Honors Theses

Student Research, Creative Works, and Publications

5-2016

Uncovering cryptic diversity of desmognathine salamanders in the Cumberland Plateau utilizing molecular genetic techniques

Katherine E. Ray
ylk161@mocs.utc.edu

Follow this and additional works at: <http://scholar.utc.edu/honors-theses>

 Part of the [Environmental Sciences Commons](#)

Recommended Citation

Ray, Katherine E., "Uncovering cryptic diversity of desmognathine salamanders in the Cumberland Plateau utilizing molecular genetic techniques" (2016). *Honors Theses*.

This Theses is brought to you for free and open access by the Student Research, Creative Works, and Publications at UTC Scholar. It has been accepted for inclusion in Honors Theses by an authorized administrator of UTC Scholar. For more information, please contact scholar@utc.edu.

Uncovering Cryptic Diversity of Desmognathine Salamanders in the
Cumberland Plateau Utilizing Molecular Genetic Techniques

Katherine Ray

Departmental Honors Thesis

University of Tennessee at Chattanooga

Biology, Geology, and Environmental Science

Project Director: Dr. Eric M. O'Neill

Examination Date: April 5, 2016

Examination Committee: Dr. Eric M. O'Neill, Dr. Yukie Kajita,

Dr. J. Hill Craddock, and Dr. Andrew Ledoan

Table of Contents

| | |
|-----------------------------|-----------|
| The Thesis | 1 |
| Abstract..... | 3 |
| Introduction..... | 4 |
| Methods..... | 7 |
| Results | 13 |
| Discussion..... | 16 |
| References | 19 |
| List of Figures..... | 23 |
| Figure 1 | 23 |
| Figure 2 | 24 |
| Figure 3 | 25 |
| Figure 4 | 29 |
| Figure 5 | 32 |
| Figure 6 | 33 |
| List of Tables | 23 |
| Table 1..... | 26 |
| Table 2..... | 27 |
| Table 3..... | 28 |
| Table 4..... | 30 |
| Table 5..... | 31 |

Abstract

Cryptic species are difficult to identify morphologically; therefore, their distributions are often poorly understood. However, species distributions are critical for evaluating biodiversity in ecology and biodiversity. Dusky salamanders (*Desmognathus*) are an example of species of North American salamanders that have been particularly difficult to classify. Specifically, in the southern Cumberland Plateau, the dusky salamanders' distributions are unknown. *Desmognathus fuscus* and *D. conanti* have been found to the north and south of the southern Cumberland Plateau, respectively. However, they have not been adequately sampled in this region. I studied genetic variation in the mitochondrial gene CytB in *Desmognathus* salamanders sampled from four locations in the southern Cumberland Plateau to attempt to determine which species is present in this area. Based on BLAST and phylogenetic analyses of the sequence data, these samples do not appear to be either *D. fuscus* or *D. conanti*, but instead are most similar to an undescribed lineage previously identified in the Smoky Mountains of Tennessee. This lineage is currently under further investigation, by other researchers, to determine if it is a new species.

Introduction

Cryptic species are generally morphologically indistinguishable but genetically distinct species (Bickford et al. 2007). While often difficult or impossible to differentiate, cryptic species generally inhabit different geographic ranges. Cryptic species occur in many types of organisms. One astounding example in particular is the neotropical skipper butterfly, *Astraptes fulgerator*. Morphological and DNA analysis of what was thought to be a single species led to the classification of ten largely allopatric species (Burns et al. 2007).

Cryptic species have been particularly significant in modern amphibian taxonomy, especially in North American Plethodontid Salamanders (Highton 2000). In several genera of the Plethodontidae, speciation has often not been accompanied by the evolution of significant morphological differences (Larson 1981; Larson 1984; Highton 1995; Wake 2009). Molecular markers, including allozymes and DNA have often been employed to distinguish between these cryptic species. For example, recent molecular analyses of the genus *Desmognathus* have revealed multiple cryptic species, especially in the Appalachian Mountains, which is a hotspot of salamander diversity (Tilley and Mahoney 1996; Mead et al. 2001; Tilley and Anderson 2003; Tilley et al. 2008; Tilley et al. 2013).

Cryptic species are important for better understanding species declines and their subsequent impacts. Current research on amphibians focuses on the issue of amphibian declines. Many declines are due to habitat loss; however, there are also many unexplainable species losses (Stuart et al. 2004). These losses were unexplainable

because the species in the area were not well documented. To better understand this troublesome issue, species of amphibians must be better documented. This documentation will allow scientists to recognize all of the species present in a given area. This creates a better understanding of the effects of amphibian declines and how conservation can be applied to these organisms. Therefore, identifying and documenting species plays a critical role in understanding biodiversity.

The dusky salamander (*Desmognathus fuscus*) was initially discovered by Constantine Samuel Rafinesque and named *Triturus fuscus* in the 19th century (Rafinesque 1820). Cope (1859) reclassified this species as a member of the genus *Desmognathus*. Rossman (1958) subsequently described the subspecies *D. fuscus conanti* as a new race of *D. fuscus* from the south-central United States. Based on a phylogenetic analysis using mitochondrial DNA, Titus and Larson (1996) found *D. fuscus* to be paraphyletic and *D. fuscus conanti* to be most closely related to *D. santeetlah*. Based on the results of their phylogenetic study and other studies of allozymes and morphology, they elevated *D. conanti* to species status.

Petranka (1998) suggested that the *Desmognathus* species complex may contain more species than previously thought. He further suggested that what was once considered *D. fuscus* could actually be subdivided into three species due to morphological differences (Petranka 1998). Bonett (2002) studied the contact zone between *D. fuscus* and *D. conanti* in western Tennessee and Kentucky (Figure 1). Based on allozyme data, Bonett (2002) concluded that in Tennessee, the Cumberland River is a possible contact zone between these species; however, no samples were included from eastern and southern Tennessee. A more recent phylogeographic study of *D. conanti* by

Beamer and Lamb (2008) focused on the coastal plain and did not include any samples of this species from Tennessee.

Currently, both *D. fuscus* and *D. conanti* are considered different species.

Desmognathus fuscus has a northern distribution extending from Maine to Eastern Kentucky and northern Tennessee (Bonett 2002; Tilley et al. 2013). *Desmognathus conanti* has a habitat range from western Tennessee to as far south as Mississippi, Louisiana, and Alabama (Bonett 2002; Beamer and Lamb 2008). While significant research has been conducted on these two species, their distributions are still not clear in central and eastern Tennessee.

The overall goals of my research were to: 1) determine which species occurs on the southern Cumberland Plateau and 2) test the hypothesis that the Tennessee River is a barrier between the two species *D. fuscus* and *D. conanti*. This area has not been sampled in previous studies. The Cumberland River appears to be a barrier between these two species in the central and western parts of northern Tennessee (Bonett 2002). Other rivers are also barrier (Tilley et al. 2013) in eastern Tennessee. To accomplish these goals, I conducted a genetic analysis on *Desmognathus* salamanders from the southern end of the Cumberland Plateau including Walden Ridge and Raccoon Mountain. Specifically, I sequenced mitochondrial DNA and compared my sequences with samples of known taxonomy in Genbank in a phylogenetic context.

Methods

Population Sampling

A total of nine unknown tissue samples (tail tips) of *Desmognathus* salamanders were collected by Eric O'Neill in the fall of 2015 from the southeastern edge of the Cumberland Plateau in Hamilton County, TN (Figures 2 and 3; Table 1). Specifically, two samples were collected from two locations (Mabbit Springs and Brimer Creek) near the Upper Chickamauga Creek on Walden Ridge, which lies north of the Tennessee River. Another two samples were collected from two locations on Raccoon Mountain (Grant Cave, Hugden Cave, and Bee creek), which lies south of the Tennessee River (Figure 3). This sampling strategy enabled me to not only potentially discover which species occurs on the southern Cumberland Plateau but also test the hypothesis that the Tennessee River is a barrier between the two species *D. fuscus* and *D. conanti*.

The samples obtained were from areas in the Cumberland Plateau and various other areas in Tennessee. The samples were from west and east Tennessee; also, samples showed the difference in distribution in areas of southern Kentucky. The samples are specifically from both sides of the Tennessee River to determine if this presents a geographical barrier to the distribution. Also, samples were obtained from Walden Ridge near Chattanooga. These samples were obtained from my field work and the sampling of other researchers in the area. Dr. Kristen Cecala (Sewanee University, Tennessee) obtained samples from the Cumberland Plateau. Samples from the Smoky Mountains were received from Mr. Justin Kratovil (University of Kentucky, Kentucky). Samples from western Tennessee were received from Dr. Ronald Bonett (University of Tulsa, Oklahoma). Thus, the samples processed represent a wide geographical range spanning

all of Tennessee and a few surrounding states. This will allow for determining what geographical barriers cause the difference in distribution of the two cryptic species.

Molecular techniques were used to effectively analyze the plethora of samples from a variety of regions. The DNA was analyzed to determine the distinctions between *D. fuscus* and *D. conanti* by comparing the mitochondrial cytochrome b region. This specific region has been previously utilized by Tilley et al. (2012) to distinguish between the two cryptic species. The research we conducted followed the model instituted by these researchers to determine if we could achieve similar results.

To confirm the accuracy of my methods and better assess the taxonomy of my unknown samples, additional samples of known taxonomy were obtained from Ronald Bonett (University of Tulsa, Oklahoma). These included three samples of *D. conanti*, two of which (Samples 118 and 119) were from Stewart County, Tennessee, which is near the alleged zone of contact between *D. fuscus* and *D. conanti*. The third sample of *D. conanti* (Sample 120) was obtained in Clarke County, GA, well outside of the range of *D. fuscus*. The known samples from Ronald Bonett also included three samples of *D. fuscus*, one from Jefferson County, IN (Sample 122), one from Simpson County, KY (Sample 128), and one from Monroe County PA (Sample 131). All three of these samples of *D. fuscus* were from areas well outside of the range of *D. conanti* and the zone of contact between *D. fuscus* and *D. conanti* (Table 2).

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted from samples using the DNeasy Tissue Kit (Qiagen)

following the manufacturer's Animal Tissues protocol. Specifically, samples were lysed and digested at 56° C for at least three hours using 180 µL of lysis buffer (Buffer ATL) and 20 µL of Proteinase K (20 mg/ml). After digestion, 200 µL of binding buffer (Buffer AL) was added to each sample. Each sample was then transferred to a DNeasy mini spin column and centrifuged. This step results in selective binding of the DNA to the DNeasy mini spin column membrane. After binding, the samples were washed twice with alcohol (Buffer AW1 and AW2) to remove remaining contaminants and enzyme inhibitors. Finally, DNA was eluted in 200 µL of elution buffer (Buffer AE) and stored at -20° C.

The polymerase chain reaction (PCR) was used to amplify a region of mitochondrial DNA prior to sequencing. PCR is a molecular technique that emulates the natural process of DNA replication used by a cell; however, the process is repeated dozens of times and resulting in millions of copies of the target region. The high copy numbers are needed for sequencing reactions. The PCR method uses oligonucleotide primers that are complementary to the 3-prime end of the target DNA region to initiate DNA replication. The enzyme *Taq*, a heat stable DNA polymerase isolated from *T. aquaticus*, creates new strands of DNA, which are complementary to the target region, from dinucleotidetriphosphates (dNTPs). Buffers are included in the reaction mixture to maintain the pH of the reaction. PCR is a three-step process that is generally repeated about 30 times. In the first step, the two DNA strands are separated from one another by raising the temperature and melting the hydrogen bonds holding the two strands together. In the second step, the temperature is lowered allowing the primers to anneal to the target DNA. The third step of PCR is extension in which dNTPs are added to created copies of the targeted region.

An approximately 400 base pair region of the mitochondrial gene cytochrome B was amplified using polymerase chain reaction (PCR) with primers CytB2 and MVZ15 (Moritz et al. 1992; Table 3). These were the same primers used by Tilley et al. (2012), which allowed me to compare my new sequence data with a larger data set from the same species. PCR was performed in a 20.0 μ L total volume: 2.0 μ L of 10x PCR buffer, 0.7 μ L of 10.0 μ M each primer, 0.4 μ L of 10.0 mM dNTPs, 0.1 μ L of 5.0 U/ μ L *Taq* polymerase, and 14.4 μ L of dH₂O. The thermocycler program included an initial 3 minute denaturation step at 95° C, 30 cycles of denaturation at 95° C for 45 seconds; then, annealing at 52° C for 45 seconds and elongation at 72° C for 30 seconds, followed by a final extension at 72° C for 5 minutes. The PCR products were visualized on 1.3% agarose gels to confirm amplification and fragment size.

Sanger Sequencing

The PCR amplified products from the samples were purified using Exo-SAP-IT following the manufacturer's protocol (USB, corp., Cleveland, Ohio, USA). Exo-SAP-IT reactions are used for PCR cleanup for downstream applications, in this case Sanger sequencing. Two hydrolytic enzymes are utilized: Exonuclease I and Shrimp Alkaline Phosphatase. The unused dNTPs and primers are removed with these enzymes. Shrimp alkaline phosphatase hydrolyzes any leftover dNTPs; while, Exonuclease I catalyzes the removal of nucleotides from single-stranded DNA in the 3' to 5' direction. Specifically, 5.0 μ L of the post PCR product was mixed with 2.0 μ L of the Exo-SAP-IT reagent and incubated at 37° C for 15 minutes. The incubation period allows for degradation of any

remaining primers and nucleotides. The next step involves another incubation at 80°C for 15 minutes to inactivate the Exo-SAP-IT reagents. Thus, Exo-SAP-IT removes these leftover dNTPs, primers, and other contaminants. At this point, the PCR products are ready for use.

To sequence the PCR products, I used the Sanger method (Sanger and Coulson 1975). This method incorporates chain-terminating dideoxynucleotides (ddNTPs) in low concentrations in a PCR. This method requires DNA primers, DNA polymerase, dNTPs, and ddNTPs. Chain terminating dideoxynucleotides do not have a 3' OH group necessary for creating a phosphodiester bond between two nucleotides. The concentration of ddNTPs is lower than the concentration of the dNTPs. The products of this method are DNA fragments of every possible length, with the 3' nucleotide fluorescently labeled with a dye specific to that nucleotide. Automated DNA- sequencing conducted using capillary electrophoresis detects the fluorescence levels. These fragments are read can then be read by a laser and a chromatogram is produced (Figure 4).

Sanger sequence reactions were conducted in the forward and reverse direction with the PCR primers described above using the Bigdye Terminator Cycle Sequencing Kit Version 1.1 (Applied Biosystems) following manufacturer protocol. Sequencing products were analyzed on an ABI PRISM 3100 capillary DNA sequencer (Applied Biosystems) at the Advanced Genetic Technologies Center (AGTC) at the University of Kentucky. Results were obtained for forward and reverse sequences. These were imported and edited using Geneious Pro version 5.3.3 (<http://www.geneious.com>; Kearse et al. 2012). Consensus sequences were generated for 9 out of 15 total samples. The nine samples included 2 known *D. fuscus* and 3 known *D. conanti*, as well as 4 unknowns.

The resulting consensus sequences were aligned using Geneious (final alignment length = 400 bp, 9 samples). All sequences will be deposited in GenBank upon completion of the project.

Data Analyses

As a first approach to identifying the species to which each sample was a member, each consensus sequence was compared with all existing GenBank sequences using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al. 1990). This search tool is useful for identifying the most similar sequences that have already been published and potentially are of known taxonomy. This tool utilizes algorithms to create effective comparisons.

As a more rigorous approach to identifying the species to which each sample was a member, a phylogeny was inferred using sequences generated in this study and 168 those from Tilley et al. (2013). Following Tilley et al. (2013), *Phaeognathus hubrichti* (The Red Hills salamander) was designated as the outgroup and *D. organi*, *D. aeneus*, and *D. quadramaculatus*, were included as more closely related basal samples. The outgroup is necessary because it provides another species not closely related to *D. fuscus* and *D. conanti*. To infer the phylogeny, Neighbor Joining (NJ) analysis with 1000 bootstrap replications (Felsenstein 1985) was performed in Geneious (Kearse et al. 2012). The best-fit model of sequence evolution used in the NJ analysis was assessed using ModelTest 3.7 (Posada and Crandall 1998).

Results

Mitochondrial DNA Sequence Variation

The nine sequences generated in this study ranged from 415 to 423 base pairs long. Sample 131 is 342 base pairs long and as not included in this range. It is an outlier in this situation because there were some sequencing complications at the ends of the region of interest. Length differences resulted from removing low quality data from the ends, which is common in Sanger sequencing. The final alignment of all nine sequences was 423 base pairs in length. A total of 335 (79.8%) sites were identical among the samples and pairwise identity was 90.8%. The known samples of *D. fuscus* and *D. conanti* were identical at 340 (99.4%) and 409 (97.4%) sites and pairwise identity was 99.4% and 98.2%, respectively. Including data from Tilley et al. (2012), the complete alignment was 426 base pairs in length, a total of 257 (60.8%) sites were identical, and the average pairwise identity was 92%.

BLAST Analysis

BLAST is the basic local alignment search tool (<http://blast.ncbi.nlm.nih.gov/Blast>). Our samples were compared to other known samples in GenBank (Table 4). The most similar sequences on Genbank are summarized in Table 4. My known *D. conanti* samples (117, 118, and 119) were most similar to *D. conanti* “isolate TAT611” (Tilley et al. 2013), which were collected from north eastern Tennessee. One known *D. fuscus* samples (122) was most similar to *D. fuscus* “haplotype B4” (Tilley et al. 2008), which were collected from the Piedmont region of Virginia. The

other known *D. fuscus* sample (133) was most similar to *D. fuscus* “haplotype B1” (Tilley et al. 2008), which were collected from from the Piedmont region of Virginia.

My unknown samples (104 and 106) were most similar to samples from the “gamma clade” described by Tilley et al. (2013), which were collected in the Great Smoky Mountains between the French Broad and Little Tennessee Rivers. Samples from this clade are currently considered innominate forms by Tilley et al. (2013), therefore they are not assigned to a species at the moment. Other unknown samples (107 and 109) were most closely related to “clade D” described by Tilley et al. (2008). These unknown samples were from the Brushy Mountains, an isolated range in the North Carolina Piedmont. Samples from this clade are also considered innominate forms by Tilley et al. (2008).

Phylogenetic Analysis

In my phylogeny, inferred using neighbor joining, all samples from the genus *Desmognathus* formed a well-supported monophyletic group (B.S. = 100) with *P. hubrichti* as the outgroup (Figures 5 and 6). Additionally, all major clades reported in Tilley et al. (2012) were found with strong support (B.S. = 75.4–97.3). As in Tilley et al. (2013), the relationships among the major clades were not well supported (B.S. < 50%). The branches are labeled with the locations names provide by Tilley et al. (2013). These location labels allow me to accurately determine which geographical location corresponds to which species.

My known *D. fuscus* samples (131 and 122) were nested within a monophyletic

group (Bootstrap = 98.8) of *D. fuscus* samples from Tilley et al. (2013). Known *D. conanti* samples 118 and 119 are nested with a monophyletic group (Bootstrap = 53.8) of *D. conanti* samples from Tilley et al. (2012). Known *D. conanti* sample 117 (Bootstrap = 88.7) was nested within a monophyletic group of *D. conanti* samples from Tilley et al. (2013).

All of my unknown samples (104,106, 107, and 109) formed a monophyletic group (Bootstrap = 95.9). Furthermore, samples 104 and 106 were sisters to one another (Bootstrap = 99.6). Unknown samples 107 and 109 are sisters to one another (Bootstrap = 100).

Discussion

The purpose of this study was to determine which species, *D. fuscus* or *D. conanti*, occurs on the southern Cumberland Plateau, and to test the hypothesis that the Tennessee River is a barrier between the two species: *D. fuscus* and *D. conanti*. I used both known and unknown samples to confirm that the molecular markers used would be able to distinguish between these species. This is part of a larger project which seeks to understand the distributions of both species in Tennessee and determine if there is a contact zone between them or if they are allopatric.

The known samples used in this study were correctly identified as either *D. fuscus* or *D. conanti* in both phylogenetic and BLAST analyses. The phylogenetic results and BLAST analysis linked the known samples with the correct species with a high degree of confidence (Table 2). These results confirm the idea that the molecular markers chosen for this study were appropriate for identifying members of these species.

From phylogenetic and BLAST analyses, it appears that all of the unknown *Desmognathus* salamanders (Mabbit Springs, Brimer Creek, Grant Cave, and Hugden Cave) in this study cannot be assigned to either *D. conanti* or *D. fuscus*, therefore neither of these species may be present on the southern Cumberland Plateau. The phylogenetic analysis was ambiguous about the exact placement of the unknown samples, but it did not suggest that these samples likely belonged to either species. However, based on the BLAST analysis, the unknown samples appear to be most similar to a recently discovered clade, the “gamma clade” that is currently under further taxonomic investigation by Stephen Tilley at Smith College (Tilley et al. 2013). Tilley was reluctant to name this

clade because of evidence of significant gene flow with other species in eastern Tennessee. They instead suggested that members of this clade be treated as innominate forms, perhaps “failed species” that began the process of speciation, but never completed it. This clade was discovered northwest of the Great Smoky Mountains between the French Broad and Little Tennessee Rivers in Tennessee. However, it appears that this clade may extend at least as far west as the Cumberland Plateau.

Overall, these results indicate that the *Desmognathus* salamanders in the southern Cumberland Plateau may be a part of the gamma clade that extends into the Great Smoky Mountains (Tilley et al. 2013). However, the phylogenetic analysis suggests that this might actually be a different undescribed species because the samples form a monophyletic group separate from all other previously described species. Further research will be necessary to determine what species is present in the southern Cumberland Plateau. Different mitochondrial genes could be studied, such as CO1 (Beamer and Lamb 2008) but studies need to involve more thorough sampling of the southern Cumberland Plateau and include more widespread samples. Thus, studying additional genetic data could provide a solution for this problem in amphibian taxonomy.

Acknowledgements

I would like to thank all individuals who have been of assistance on this project. Dr. O'Neill has been invaluable in teaching me about the lab. Dr. Kajita has also provided much assistance in regards to molecular techniques. I would also like to thank members of my DHON committee: Dr. Ledoan and Dr. Craddock, for providing constructive criticism for my project. Lastly, I would like to thank Lily and Coleman for providing support and encouragement throughout this process.

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. and Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecol. Evol.* 22(3), pp.148-155.
- Bonett, R. (2002). Analysis of the contact zone between the dusky salamanders *Desmognathus fuscus* and *Desmognathus conanti*. *Copeia*. 2002: 344-355.
- Beamer, D and Lamb, T. (2008). Dusky salamanders (*Desmognathus*, Plethodontidae) from the Coastal Plain: multiple independent lineages and their bearing on the molecular phylogeny of the genus. *Mol. Phylogenet. Evol.* 143-153.
- Burns, J., Janzen, D., Hajibabaei, M., Hallwachs, W., and Herbert, P. (2007). DNA barcodes and cryptic species of skipper butterflies in the genus *Perichares* in Area de Conservacion Guanacaste, Costa Rica. *Proc. Natl. Acad. Sci. U.S.A.* 105(17): 635-6255.
- Cope, E.D. (1859). On the primary divisions of the Salmadridae, with descriptions of two new species. *Proc. Natl. Acad. Sci. Philadelphia*. 11: 122-128.
- Felsenstein, J. (1985). Phylogenies and the Comparative Method. *Amer. Nat.* 125(1): 1-15.
- Highton, R. (1995). Speciation In eastern North American salamanders of the genus *Plethodon*. *Annu Rev Ecol Syst.* 26:579-600.
- Highton, R. (2000). Detecting cryptic species using allozyme data. In *The biology of plethodontid salamanders* (pp. 215-241). Springer US.

- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., and Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 28(12), 1647-1649.
- Kozak, K. H., Larson, A., Bonett, R. M., & Harmon, L. J. (2005). Phylogenetic analysis of ecomorphological divergence, community structure, and diversification rates in dusky salamanders (Plethodontidae: *Desmognathus*). *Evol.* 59(9): 2000-2016.
- Kozak K., Mendyk R., and Wiens J. (2009). Can parallel diversification occur in sympatry? Repeated patterns of body- size evolution in coexisting clades of North American salamanders. *Evol.* 63(7):1769-1784.
- Larson, A., D. B. Wake, L. R. Maxson, and R. Highton. (1981). A molecular phylogenetic perspective on the origins of morphological novelties in the salamanders of the tribe Plethodontini (Amphibia, Plethodontidae). *Evol.* 35: 405-422.
- Larson, A. (1984). Neontological inferences of evolutionary pattern and process in the salamander family Plethodontidae. Pp. 119-127 in M.K. Hecht, B. Wallace and G.T. Prance (editors). *Evol. Biol.* Volume 17. Plenum Publ. Co. New York.
- Larson, A., D. W. Weisrock, and K. H. Kozak. (2003). Phylogenetic systematics of salamanders (Amphibia: Urodela), a review. Pp. 31-108 in Reproductive Biology and Phylogeny of Urodela (D. M. Sever, ed.) Science Publishers, Inc., Enfield (NH), USA.
- Mead, L.S., Tilley, S.G. & Katz, L.A. (2001). Genetic structure of the Blue Ridge dusky

- salamander (*Desmognathus orestes*): inferences from allozymes, mitochondrial DNA, and behavior. *Evol.* 55: 2287–2302.
- Petranka J. W. (1998). Salamanders of the United States and Canada. Washington: Smithsonian Institute Press.
- Pilliod D., Goldberg C., Laramie M., and Waits L. (2013). Application of Environmental DNA for Inventory and Monitoring of Aquatic Species. U.S. Geological Survey.
- Posada D. and Crandall K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics.* 14(9): 817-818.
- Rafinesque, C. S. (1820). *III Class. Erpetia- The reptiles.* Annals of Nature, or, Annual Synopsis of New Genera and Species of Animals, Plants, etc. Discovered in North America. Lexington, Kentucky 1: 4-6.
- Sanger, F. and Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* 25; 94(3): 441–448.
- Stuart S., Chanson J., Cox N., Young B., Rodrigues A., Fishman D., and Waller R. (2004). Status and trends of Amphibian declines and extinctions worldwide. *Science Report.*
- Tilley, S. G. and Mahoney, M. J. (1996). Patterns of genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Amphibia: Plethodontidae). *Herpetol Monog.* 10: 1-42.
- Tilley, S. G. and Anderson J. (2003). Systematics of the *Desmognathus ochrophaeus* complex in the cumberland plateau of tennessee. *Herpetol Monog.* 151: 75-110.

- Tilley S., Eriksen R., and Katz L. (2008). Systematics of Dusky Salamanders, *Desmognathus*, in the mountain and piedmont regions of Virginia and North Carolina, USA. *Zool. J. Linn.* 152: 115-130.
- Tilley S., Bernardo J., Katz L., Lopez L., Roll D., Eriksen R., Kratvil J., Bittner N., and Crandall K. (2013). Failed Species, innominate forms, and the vain search for species limits: cryptic diversity in dusky salamanders (*Desmognathus*) of Eastern Tennessee. *Ecol Evol.* 3(8): 2547-2567.
- Titus, T. A. and Larson, A. (1996). Molecular phylogenetics of desmognathine salamanders (Caudata: Plethodontidae): a reevaluation of evolution in ecology, life history, and morphology. *Syst Biol.* 45(4): 451.
- Wake, D. B., and C. J. Schneider. (1998). Taxonomy of the plethodontid salamander genus *Ensatina*. *Herpetologica.* 54: 279–298.
- Wake, D.B. (2009). What Salamanders Have Taught Us about Evolution. *Annu. Rev. Ecol. Evol. Syst.* 40: 333-352.

Figure 1. Depiction of the zone of contact between *D. conanti* and *D. fuscus*.

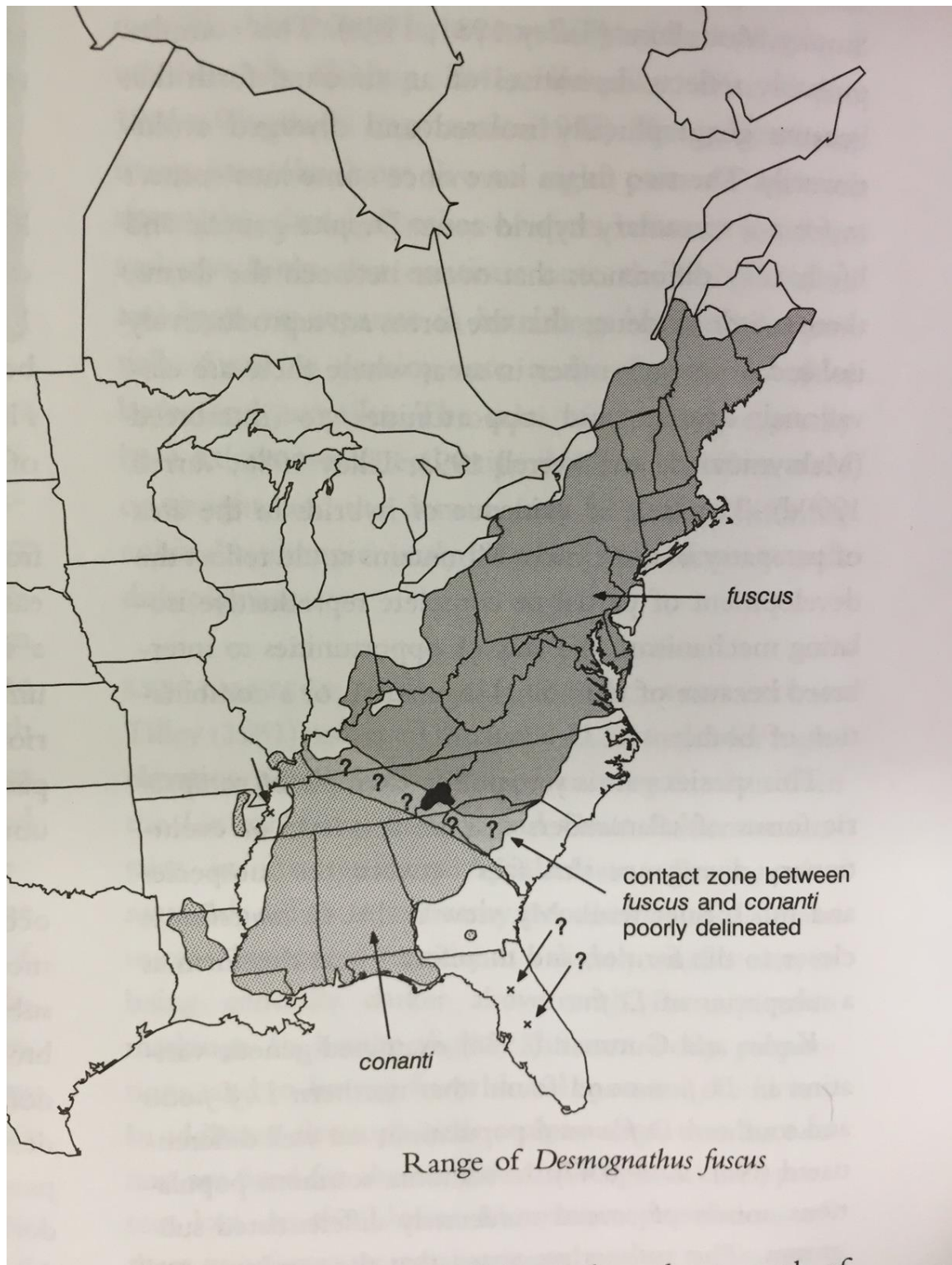


Figure 2. Species distribution in the United States.

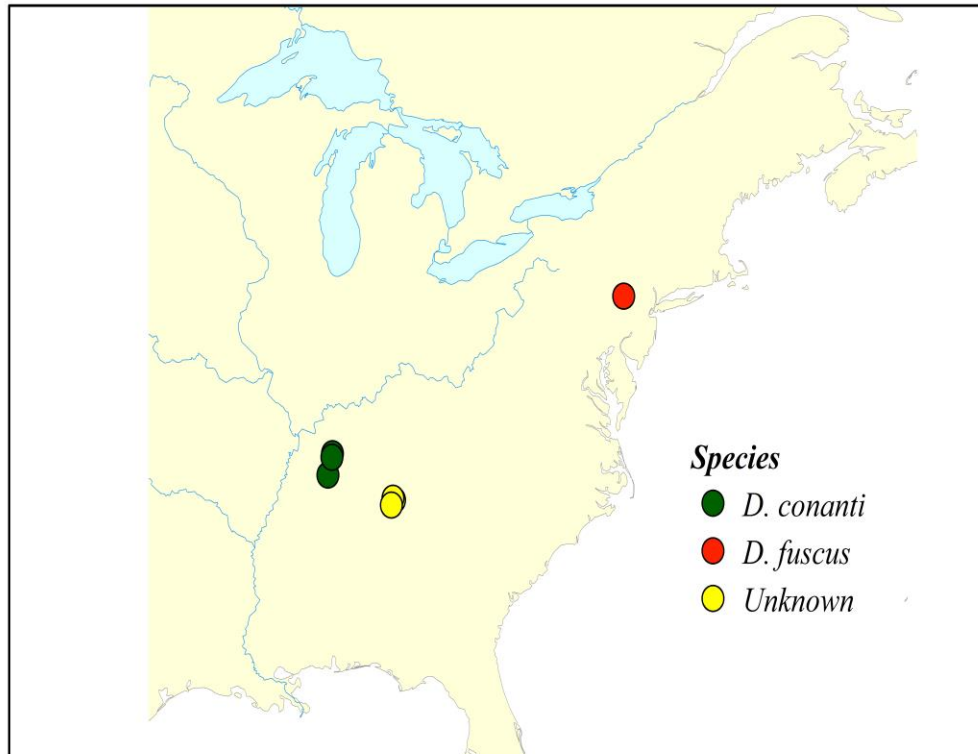


Figure 3. Species distribution in the southern Cumberland plateau

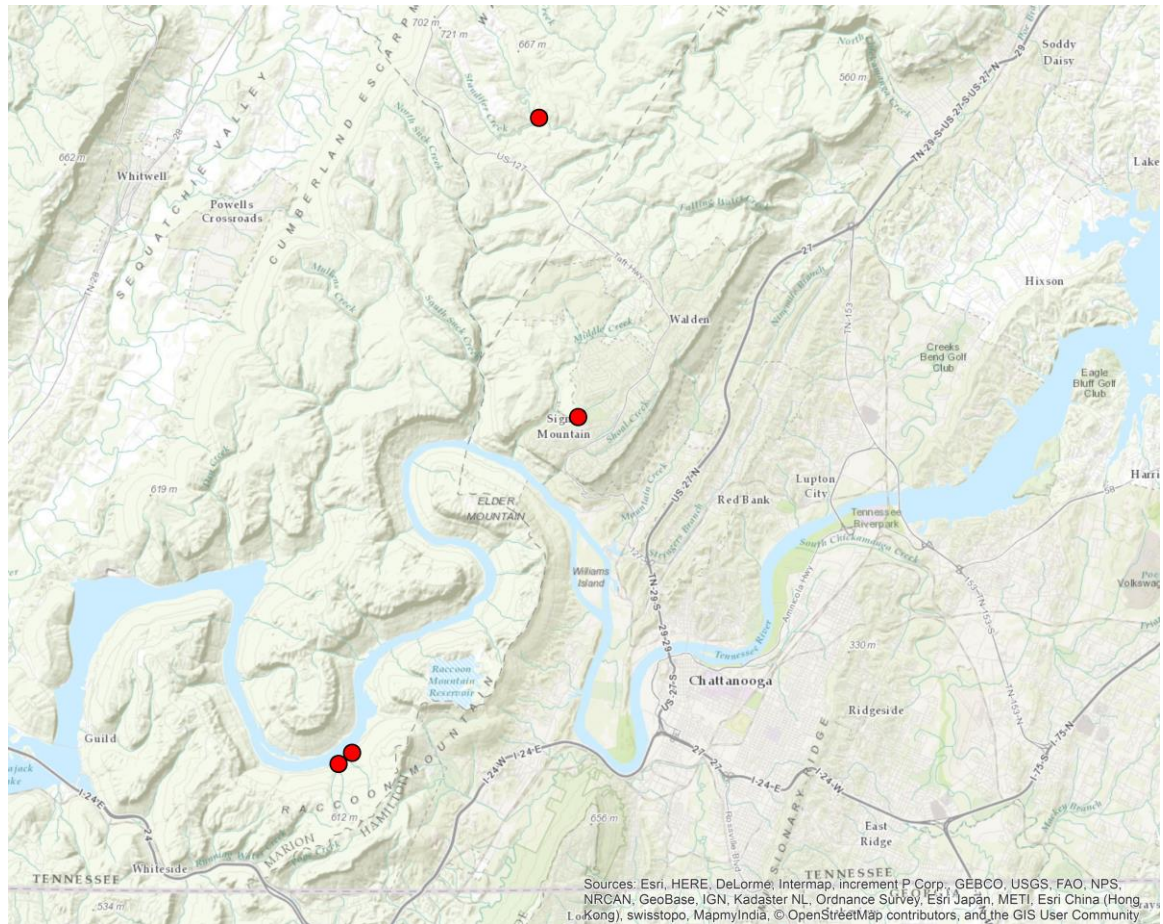


Table 1. Samples sequenced in this study, species identity when known, and location.

| Sample Number | Species | Location Collected |
|----------------------|-------------------|---------------------------|
| 104 | Unknown | Mabbit Springs, TN |
| 106 | Unknown | Brimer Creek, TN |
| 107 | Unknown | Grant Cave, TN |
| 109 | Unknown | Hugden Cave, TN |
| 110 | Unknown | Hugden Cave, TN |
| 116 | <i>D. conanti</i> | Tishamigo County, MS |
| 117 | <i>D. conanti</i> | Benton County, TN |
| 118 | <i>D. conanti</i> | Steward County, TN |
| 119 | <i>D. conanti</i> | Stewart County, TN |
| 120 | <i>D. conanti</i> | Clarke County, GA |
| 122 | <i>D. fuscus</i> | Jefferson County, IN |
| 128 | <i>D. fuscus</i> | Simpson County, KY |
| 131 | <i>D. fuscus</i> | Monroe County, PA |
| 132 | <i>D. fuscus</i> | Cannon County, TN |
| 133 | <i>D. fuscus</i> | Cumberland County, TN |

Table 2. Known samples

| Sample Number | Sample Name | Specific Location |
|----------------------|--------------------|--------------------------|
| 118 | <i>D. conanti</i> | Stewart County, TN |
| 119 | <i>D. conanti</i> | Stewart County, TN |
| 120 | <i>D. conanti</i> | Clarke County, GA |
| 122 | <i>D. fuscus</i> | Jefferson County IN |
| 131 | <i>D. fuscus</i> | Monroe County, PA |

Table 3. Primers utilized in PCR and sequencing.

| Primer | Sequence | Source |
|---------------|--------------------------------|--------------------|
| Mvz15 | 5' GAACTAATGGCCCACACWWTACG 3' | Moritz et al. 1992 |
| Cytb2 | 5'CCCCTCAGAATGATATTTGTCCTCA 3' | Moritz et al. 1992 |

Figure 4. Chromatogram from Sanger sequencing method.

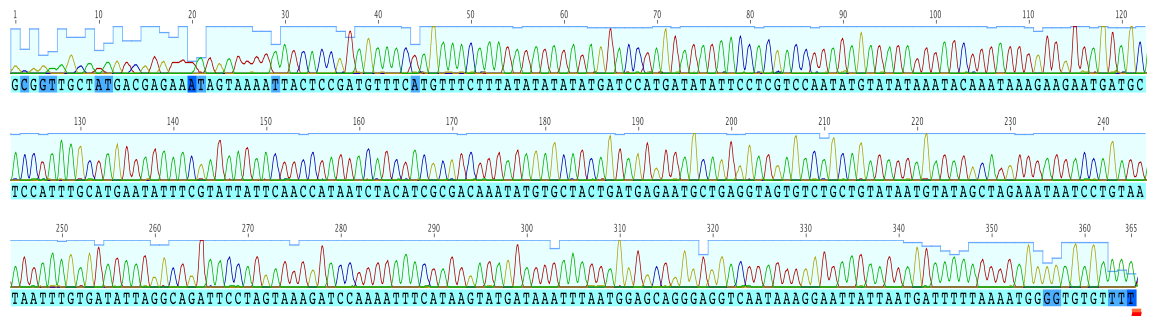


Table 4. BLAST analysis

| Sample | Closest Match | GeneBank Accession # | Maximum Identity Score (%) |
|---------------|--------------------------------------|---------------------------------|---------------------------------------|
| 104 | Desmognathus sp. ‘gamma clade’ | KF242407 | 92 |
| 106 | Desmognathus sp. ‘gamma clade’ | KF242407 | 92 |
| 107 | Desmognathus sp. D2 Cytochrome B | EF028660 | 91 |
| 109 | Desmognathus sp. D2 Cytochrome B | EF028660 | 91 |
| 117 | D. conanti isolate TAT611 cytochrome | KF242413 | 99 |
| 118 | D. conanti isolate TAT611 cytochrome | KF242414 | 99 |
| 119 | D. conanti isolate TAT611 cytochrome | KF242414 | 99 |
| 122 | D. fuscus mitochondrion | AY728227 | 99 |
| 131 | D. fuscus haplotype B1 cytochrome B | EF028650 | 99 |

Table 5. Information from sequenced samples.

| Sample Name | Length in base pairs | Identical sites (%) | Pairwise Identity % | GC content | %A | %C | %T | %G |
|--------------------|-----------------------------|----------------------------|----------------------------|-------------------|-----------|-----------|-----------|-----------|
| 104 | 415 | 79.5 | 90.8 | 32.1 | 32.9 | 18.1 | 34.9 | 14.1 |
| 106 | 415 | 79.5 | 90.8 | 32.5 | 33.0 | 18.1 | 34.5 | 14.5 |
| 107 | 419 | 79.7 | 90.8 | 34.1 | 31.3 | 18.9 | 34.6 | 15.3 |
| 109 | 423 | 79.8 | 90.8 | 34.3 | 31.4 | 18.4 | 34.3 | 15.8 |
| 117 | 420 | 79.8 | 90.8 | 33.3 | 29.8 | 17.4 | 16.0 | 36.9 |
| 118 | 420 | 79.8 | 90.8 | 33.1 | 30.7 | 17.9 | 15.2 | 36.4 |
| 119 | 418 | 79.7 | 90.8 | 33.3 | 30.4 | 17.9 | 15.3 | 36.4 |
| 122 | 420 | 79.8 | 90.8 | 31.9 | 32.1 | 17.6 | 14.3 | 36.0 |

Figure 5. Phylogenetic tree with data from this study and Tilley et al. (2012).

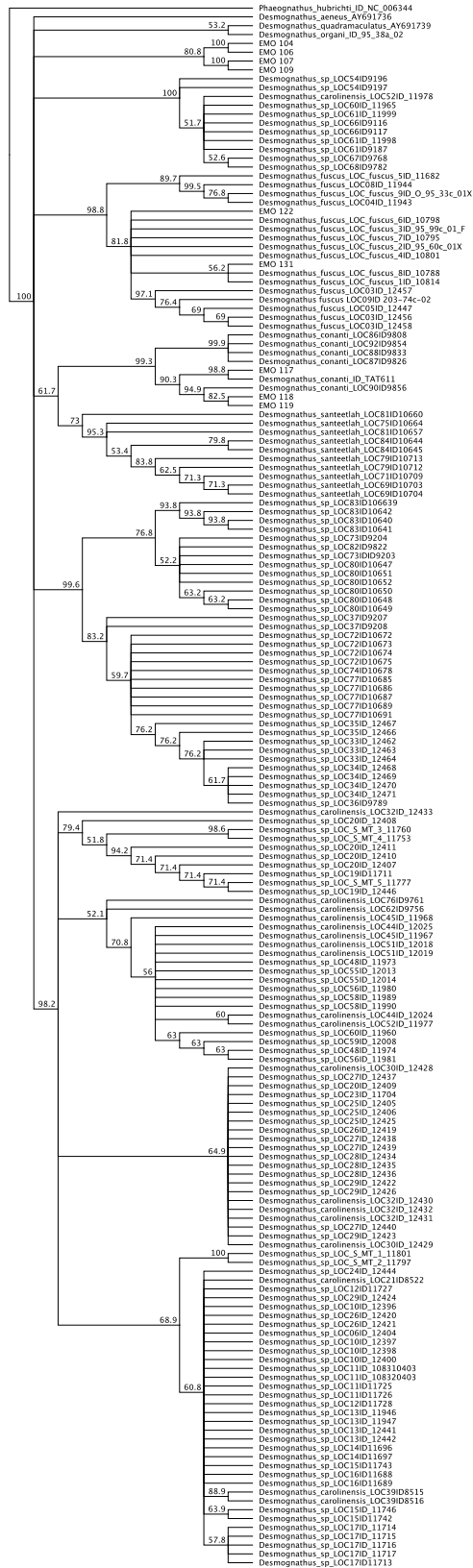


Figure 6. A reduced tree with data from this study and a representative set of samples from Tilley et al. (2012).

